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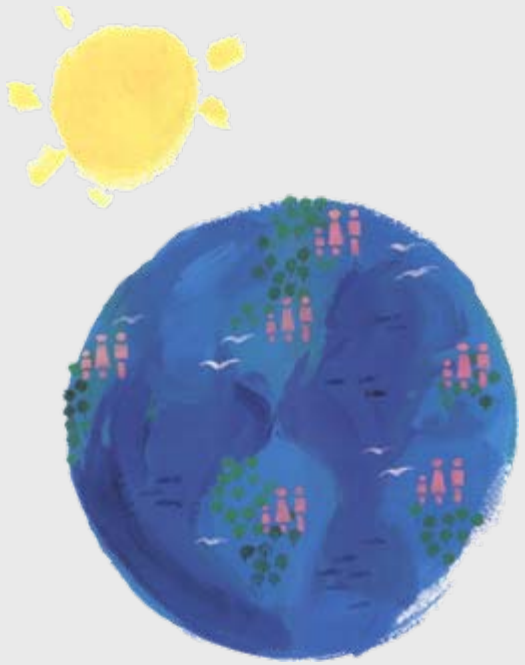
National Toxicology Program
Interagency Center for the Evaluation of Alternative Toxicological Methods

ICCVAM

Interagency Coordinating Committee on the Validation of Alternative Methods



Overview of the LLNA Independent Scientific Peer Review Panel Report

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Charge to the Peer Panel

- Review the ICCVAM Draft Background Review Documents (BRDs) for completeness and identify any errors or omissions in the BRDs.
- Evaluate the extent to which applicable validation and acceptance criteria of toxicological test methods (ICCVAM 2003) have been appropriately addressed.
- Consider the ICCVAM draft test method recommendations for the following and comment on the extent to which they are supported by the information provided in the BRD:
  - Proposed test method uses and limitations
  - Proposed recommended standardized protocols
  - Proposed test method performance standards
  - Proposed future studies

LLNA Modifications and Applications Evaluated

1. LLNA Limit Dose Procedure
2. LLNA for testing Mixtures, Metals, and Aqueous Solutions
3. Non-Radiolabeled LLNA Test Methods:
   - LLNA: DA measures cell proliferation by measuring ATP levels
   - LLNA: BrdU-FC measures cell proliferation by detecting BrdU via flow cytometry
   - LLNA: BrdU-ELISA measures cell proliferation by detecting BrdU via an ELISA
4. Draft ICCVAM LLNA Performance Standards
5. Use of the LLNA for Potency Determinations

These are abbreviated highlights of the final Independent Scientific Peer Review Panel report. The final report should be consulted for a detailed description of the Panel's conclusions and recommendations.

1. LLNA Limit Dose Procedure

- Follows the traditional ICCVAM LLNA protocol except for the number of doses tested
  - Uses only the high dose group (requires 40% fewer animals)
- In general, the Panel concurred with the draft ICCVAM recommendation that the LLNA limit dose procedure should be routinely recommended for the hazard identification of skin sensitizing chemicals when dose response information is not required.
- The Panel also recommended that if dose response information information is required, the LLNA limit dose procedure should be routinely recommended as the initial test to identify sensitizers before conducting the traditional LLNA as a way to further reduce animal use since negative results would not require further testing.
- Also suggested that the test be referred to as the rLLNA ("reduced LLNA") to be consistent with ECVAM terminology.

2. LLNA Applicability Domain

- The Panel agreed with the draft ICCVAM recommendations for using the LLNA to test mixtures, metals, and substances tested in aqueous solutions. That is:
  - *Mixtures* - More data are needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures can be made.
  - *Metals* - The LLNA appears useful for the testing of metal compounds, with the exception of nickel.
  - *Substances tested in aqueous solutions* - More data are needed before a recommendation on the usefulness and limitations of the LLNA for testing substances in aqueous solutions can be made.
- Based on limitations inherent in the available data set, the Panel emphasized the need for the continued accrual of information (i.e., LLNA data, comparative guinea pig and human data) for mixtures, metals, and substances tested in aqueous solutions.

3. Non-Radiolabeled LLNA Protocols

- In general, the Panel agreed with the draft ICCVAM recommendations that the three non-radiolabeled LLNA protocols may be useful for identifying substances as potential skin sensitizers and non-sensitizers.
  - Existing validation study data and results need to be provided and examined before these methods can be recommended for use.
  - The number of animals per dose group should be based on power calculations.

LLNA: DA

- The LLNA: DA may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but this recommendation is contingent upon receipt of additional data and information.
  - Pretreatment with 1% SLS should not be accepted until its impact on the performance of the LLNA: DA has been adequately characterized.
    - 1% SLS pretreatment should not be recommended unless it is proven to have no immunomodulatory effect in the draining auricular lymph nodes or alter the sensitivity of the application site to irritants.
  - It should be demonstrated that the treatment schedule does not elicit an immune response beyond the induction phase of allergic contact dermatitis (i.e., the elicitation phase).

LLNA: BrdU-FC

- The LLNA: BrdU-FC may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but this recommendation is contingent upon receipt of additional data and information.
  - 3 out of 18 substances (17%) produced a false positive response compared to the traditional LLNA.
    - Since only a single laboratory is using the LLNA: BrdU-FC, the Panel recommended that the raw data be made available and that additional studies in the LLNA: BrdU-FC determine if the initial results are repeatable.
  - The Panel emphasized the need to conduct interlaboratory studies as part of the validation process.

LLNA: BrdU-ELISA

- The LLNA: BrdU-ELISA may be useful for identifying substances as potential skin sensitizers and non-sensitizers.
- However, more data and information are needed before the method is recommended.
  - A detailed protocol is needed.
  - The current database is not sufficiently robust.
    - Sufficient quantitative data are needed to allow for broader analysis on a larger set of balanced reference substances.
  - Concern about using a decision criteria of SI ≥1.3, instead of SI ≥ 3.0, to optimize test performance.
    - Power calculations indicate an increase in the number of animals required to obtain acceptable confidence interval at SI ≥1.3.
    - It may be more appropriate to use a statistically based decision criteria than a ratio.

4. Draft ICCVAM LLNA Performance Standards: Essential Test Method Components

- The Panel agreed that the use of non-radioactive reagents for measuring cell proliferation is a “minor” modification of the traditional LLNA protocol.
- However, other modifications may be “minor”.
  - A better strategy for the performance standards might be to define criteria that need to be satisfied to insure that the method is mechanistically and functionally similar and that the performance of the modified LLNA protocol is considered adequate relative to the traditional LLNA.
- The proposed performance standards are robust.
  - Regardless of modification (i.e., “major” or “minor”), there is the same expectation for evaluation of performance.
- Examples of acceptable modifications as long as adequate performance to the traditional LLNA is demonstrated and the method is mechanistically and functionally similar:
  - Sex, strain, species, animals per group, timing of test article treatment.
- The test method must only measure the induction phase of the immune response only.

Draft ICCVAM LLNA Performance Standards: Essential Test Method Components (2)

- During validation of a modified LLNA, data must be collected at the level of individual animals to allow an estimate of the variance within control and treatment groups.
- Until data are collected to enable a reliable power calculation to be conducted and a sufficient number of animals per dose group determined, at least five mice per dose group should be used.
- A concurrent positive control should be run with each test substance to ensure that the system is operating as expected and technical errors are not occurring.
  - If a known sensitizer is being tested during the validation effort, a concurrent positive control might not be needed.
- Once the revised test method has been adequately validated, a concurrent positive control is recommended unless the laboratory has extensive historical data indicating that the positive control consistently yields statistically bioequivalent results in the modified LLNA assay under testing.
  - Then, on a regular periodic basis, evaluation of a positive control should be recommended.

Draft ICCVAM LLNA Performance Standards: Accuracy Standards

- The current database does not support the inclusion of EC3 values as a component of the accuracy evaluation.
- For use in hazard identification, a modified method should be evaluated with all 22 substances on the ICCVAM list (including the 4 optional substances) and accuracy statistics calculated.
  - Ideally, an alternative LLNA protocol should be equivalent to the traditional LLNA.
    - However, with the small number of reference substances available, establishing equivalence will be extremely difficult.
  - Therefore, it may not be necessary to reach the same level of accuracy if appropriate rationale for any discordance is provided.
    - The sensitizers on the list should be weighted such that the strongest sensitizers are not allowed to be missed.
    - Considerable weight will be given to the balance between animal welfare and human safety when considering the adequacy of test method accuracy.

Draft ICCVAM LLNA Performance Standards: Reliability Standards

- The Panel considered using the ECt range to be appropriate for the intralaboratory reproducibility analysis because a large database of LLNA studies is available for HCA and the multiple comparison problem[1] does not exist.
- Similarly, the Panel considered use of the ECt range to be appropriate for the interlaboratory reproducibility analysis because a large database of LLNA studies is available for HCA and DNCB.

[1]When multiple experiments are conducted and multiple observations, comparisons or hypothesis tests are conducted, the chance of observing rare events increases. Suppose, for example, that an interval is established such that 5% of observations from a particular population of data are outside that interval. Then if *k* independent experiments generate data from this population (e.g., a standard normal distribution), the chances that all 20 results will lie inside the interval is (1.0 - 0.05)*k* (N. Flournoy, personal communication).

Draft ICCVAM LLNA Performance Standards: Reference Substances

- The appropriateness of the 0.5x to 2.0x EC3 range for the reference substances has not been adequately justified.
  - It is certainly not appropriate to include chemicals represented by only one LLNA study in a list of recommended reference substances, as there is insufficient data for which to define a mean EC3 value.
- Thus, these compounds should either be:
  - Exchanged for compounds with sufficient EC3 data (i.e., based on ≥ 3 studies) using the same solvent, or
  - Retained but not considered to be part of the EC3 criterion until such data has been collected.

5. Use of the LLNA for Skin Sensitization Potency Determinations

- The Panel agreed with the ICCVAM recommendation that the LLNA should not be considered a stand-alone assay for categorization of skin sensitization potency, but it could be used in a weight-of-evidence evaluation to discriminate between strong and weak sensitizers.
- More data are needed to determine the optimal threshold in humans for distinguishing between strong and weak sensitizers.
  - No new animal studies should be conducted unless it is likely that such studies will lead to an overall reduction in animal use.

Peer Review Panelists

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- Sidney Green, Ph.D. - Howard University College of Medicine, USA
- **Kim Headrick, B. Admin., B.Sc. - Health Canada, Canada (Evaluation Group Chair)**
- Dagmar Jírová, M.D., Ph.D. - National Institute of Public Health, Czech Republic
- David Lovell, Ph.D. - University of Surrey, United Kingdom
- Michael Luster, Ph.D. - Consultant to NIOSH Health Effects Laboratory, USA (Panel Chair)
- Howard Maibach, M.D. - University of California - San Francisco, USA
- James McDougal, Ph.D. - Wright State University, USA
- **Michael Olson, Ph.D. - GlaxoSmithKline, USA (Evaluation Group Chair)**
- Raymond Pieters, Ph.D. - Utrecht University, Netherlands
- Jean Regal, Ph.D. - University of Minnesota, USA
- Jonathan Richmond, MB ChB, FRCSEd - Animals Scientific Procedures Division, United Kingdom
- Peter Theran, V.M.D. - Massachusetts Society for the Protection of Cruelty to Animals, USA
- Stephen Ullrich, Ph.D. - MD Anderson Cancer Center, USA
- **Michael Woolhiser, Ph.D. - Dow Chemical Company, USA (Evaluation Group Chair)**
- Takahiko Yoshida, M.D., Ph.D. - Asahikawa Medical College, Japan

1. Do you have any comments on the panel's conclusions and recommendations on the five draft ICCVAM BRDs, the draft ICCVAM BRD Addendum, and the draft LLNA Performance Standards in regard to their completeness and any identified errors or omissions?
2. Do you have any comments on the panel's conclusions and recommendations in terms of the extent to which each of ICCVAM's applicable criteria for validation and acceptance of alternative test methods have been addressed appropriately in each draft test method BRD or the draft BRD Addendum?
3. Do you have any comments on the draft ICCVAM test method recommendations for the seven LLNA methods and applications?
4. Do you have any comments on the panels comments, conclusions, or recommendations for the LLNA methods and applications regarding:
   a. their usefulness and limitations?
   b. the recommended test method protocols?
   c. test method performance standards?
   d. the proposed additional studies?
5. Do you have any comments on the panel's comments, conclusions, or recommendations regarding the ICCVAM draft LLNA performance standards?